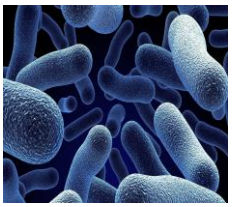
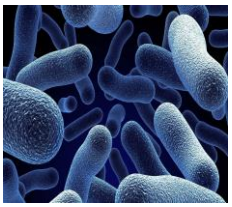


Microbiology Method On The basis of IS and ISO Standard

TITLE: SOP FOR MICROBIOLOGY LAB.			
Document Name:	Standard Operating Procedure	Effective Date	09/11/2017
Document Number		Issue Date	
Department:	Quality control- Microbiology		

Prepared By:- Mr. Vinay Katare	**Checked By: Mr. Abhishek Garhewal**	**Approved By:-**



Microbiology Method On The basis of IS and ISO Standard

TITLE: SOP FOR MICROBIOLOGY LAB.			
Document Name:	Standard Operating Procedure	Effective Date	09/11/2017
Document Number		Issue Date	
Department:	Quality control- Microbiology		

1.0 PURPOSE- Enumeration of micro-organism **COLONY COUNTS TECHNIQUE** at 30°C [total plate count]

2.0 SCOPE: To Enumeration of micro-organism Colony Counts Technique at 30°C [total Plate Count]

3.0 RESPONSIBILITY: Microbiologist

4.0 REFERENCES: IS 5402:2002/ ISO 4833:1991

5.0 DEFINITIONS: - This test gives an indicator of mesospheric aerobic microorganism's count of product and can give an indicator of general hygiene of product.

6.0 APPARATUS AND GLASSWARE :- Petri plate, Incubator, Autoclave, Test-tube, Water bath, pH meter, Colony Counting Equipment, Laminar Air Flow Chamber, Micro Pipette, etc.

7.0 CULTURE MEDIA:-Plate Count Agar (M019) final pH at 25°C 7.2±0.2 Buffer Peptone Water (M614) final pH at 25°C 7.2±0.2 sterilization is carried out by autoclave at 121 °C for 15 minutes at 15 lbs.

8.0 PROCEDURE:- Test portion initial suspension and dilution –Take 10 gm of sample and 90 ml of diluent buffer peptone water.

9.0 INOCULATION AND INCUBATION:-

- Take two sterile Petri dishes using a sterile micropipette tips transfer to each dish 1 ml of the test sample if the product is liquid or 1ml of the initial suspension in the case of other products.
- Take two other sterile Petri dishes using a fresh sterile micro tips transfer to each dish 1 ml of the first decimal dilution (10-2) of the initial suspension in the

case of other product repeat the procedure with the further dilutions using a fresh sterile micro tips for each decimal dilution.

- Pour about 15 ml of the PCA medium at 45 °C ±0.5 C in to each Petri dish the time eclipsing between the ends the preparation of the initial suspension (or of the 10^{-1} dilution if the product is liquid) Invert the prepared dishes and invert them in the incubator set 37 °C for 48 hrs.

10.0 COUNTING THE COLONIES:-After the specified period of inoculation count using the colony counting unit equipments the colonies in each dish containing not more than 300 colonies.

11.0 EXPRESSION OF RESULT:-

- General case dishes containing between 15 and 300 colonies.
- Retained dishes containing not more than 300 colonies at two consecutive dilutions it is necessary that one of these dishes contain at least 15 colonies.
- Calculate the number N of microorganism per milliliter or per gram of the product depending on the case using the following equation.

$$N = \frac{\Sigma C}{(n_1 + 0.1 n_2)\, d}$$

Where

Σc is the sum of colonies contends on all the dishes retained.

n_1 is the number of dishes retained in the first dilution.

n_2 is the number of dishes retained in the second dilution.

d is the dilution factor corresponding to the first dilution.

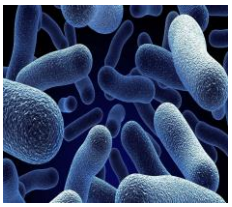
Round the result calculated to two significance figures

Take as the number of microorganisms per milliliter or per gram of product expressed as a number between 1.0 and 9.9 multiplied by 10X is the appropriate power of 10.

12.0 TESTING FREQUENCY:-

- For raw material per batch.
- For online material; every lot (Inprocess).
- For finished material every batch.

13.0 ATTACHMENT –Nil



Microbiology Method On The basis of IS and ISO Standard

TITLE: SOP FOR MICROBIOLOGY LAB.			
Document Name:	Standard Operating Procedure	Effective Date	09/11/2017
Document Number		Issue Date	
Department:	Quality control- Microbiology		

1.0 PURPOSE:- Method for **YEAST AND MOULD COUNT** of foodstuffs and animal feeds.

2.0 SCOPE:-Method for **YEAST AND MOULD COUNT** of foodstuffs and animal feeds**.**

3.0 RESPONSIBILITY:-Microbiologist.

4.0 REFERENCES:-IS 5403:1991

5.0 DEFINITIONS:-This method describes Yeast and Mould count expressed number of colonies as per gm/ml.

6.0 AAPRATUS AND GLASSWARE:-Petri plate, Incubator, Aautoclave, Test-tube, Water bath, pH meter, Colony Counting Equipment, Laminar Air Flow Chamber, Micro Pipette etc.

7.0 CULTURE MEDIA:-Potato Dextrose Agar(M096)/Yeast Glucose Chloramphenicol Agar (M1590) final pH at 25 °C 5.6± 0.2 Buffer Peptone Water (M614) final pH at 25°C 7.2± 0.2 Sterilization is carried out by autoclave at 121 °C for 15 minutes at 15 lbs.

8.0 PROCEDURE:-Test portion initial suspension and dilution –Take 10 gm of sample and 90 ml of diluent Buffer Peptone Water.

9.0 INOCULATION AND INCUBATION:-

9.1 Take two sterile Petri dishes using a sterile Micropipette tips transfer to each dish 1 ml of the test sample.

9.2 If the product is liquid or 1ml of the initial suspension in the case of other products Take two other sterile Petri dishes using a fresh sterile micro tips transfer to each dish 1 ml of the first decimal dilution (10^{-2}) of the test sample if the product is liquid or 1 ml of the first decimal dilution (10^{-2}) of

the initial suspension in the case of other products repeat the procedure with the further dilutions using a fresh sterile micro tips for each decimal dilution.

9.3 Pour about 15 ml of the PDA medium/ Yeast Glucose Chloramphenicol Agar at 45 °C ±0.5 °C in to each Petri dish the time eclipsing between the ends the preparation of the initial suspension (or of the 10^{-1} dilution if the product is liquid) Invert the prepared dishes and invert them in the incubator set 25°C for 3, 4, 5 days.

10.0 COUNTING THE COLONIES:-After the specified period of inoculation count using the colony counting unit equipments the colonies in each dish containing not more than 300 colonies.

11.0 EXPRESSION OF RESULT:-General case dishes containing between 15 and 300 colonies. Retained dishes containing not more than 300 colonies at two consecutive dilutions it is necessary that one of these dishes contain at least 15 colonies. Calculate the number N of microorganism per milliliter or per gram of the product depending on the case using the following equation.

$$N = \frac{\Sigma C}{(n_1 + 0.1 n_2)\, d}$$

Where

ΣC is the sum of colonies contends on all the dishes retained.

n_1 is the number of dishes retained in the first dilution.

n_2 is the number of dishes retained in the second dilution.

d is the dilution factor corresponding to the first dilution.

Round the result calculated to two significance figures

Take as the number of microorganisms per milliliter or per gram of product expressed as a number between 1.0 and 9.9 multiplied by 10X is the appropriate power of 10.

12.0 TESTING FREQUENCY:-

12.1 For raw material per batch.

12.2 For online material; every lot (In process).

12.3 For finished material every batch.

13.0 ATTACHMENT –Nil

1.0 PURPOSE:- Method for Isolation and Detection and Identification of **Escherichia coli** (Responsible for Food Poisoning).

2.0 SCOPE:-Method for Isolation and Detection and Identification of **Escherichia coli** (responsible for food poisoning).

3.0 RESPONSIBILITY:- Microbiologist.

4.0 REFERENCES: IS 5887:1976

5.0 DEFINITIONS:-The typical **Escherichia coli** is aerobic, gram negative, rod shaped, motile, fermenting with the gas production and usually produces smooth colony non mucoid colonies on solid media however there are non lactose fermenting strain of E.coli and some strain produce mucoid colony.

6.0 AAPRATUS AND GLASSWARE:- Screw cap tube, Inoculating loop, Petri plate, Incubator, Autoclave, Test-tube, Water bath, pH meter, Colony Counting Equipment, Laminar Air Flow Chamber, Micro Pipette etc.

7.0 CULTURE MEDIA:- MacConkey agar(M081B), MacConkey Broth(MH083), Kovacs reagent(R008),Peptone Water(M028),LST broth (M080) EC broth (M127) final pH at 25 °C 6.9± 0.2, EMB Agar (M317) final pH at 25°C 7.2± 0.2 Sterilization is carried out by Autoclave at 121°C for 15 minutes at 15 lbs.

8.0 PROCEDURE:-Test portion initial suspension and dilution take 10 gm of sample and 90 ml of diluents buffer peptone water.

9.0 INOCULATION AND INCUBATION:-

- Inoculate 1 ml of the blended or macerated sample into 10 ml of single strength MacConkey broth medium. And incubate at 37°C for 24 hour.
- After incubation gas formation is positive in tube proceed in selective media.
- If the number of organisms is assumed to be very small, inoculate 10 ml of double strength MacConkey broth medium.(And incubate at 37°C for 24 hour. After incubation gas formation is positive in tube proceed in selective media.)
- If there is growth with fermentation of lactose in the MacConkey broth medium streak out a loopful on to each of the solid selective media like MacConkey agar medium, Eosin Methylene Blue Lactose agar , if available Tergitol-7 agar and incubate all the plates at 37°C overnight.
- On EMB if E.coli is grown it will give a distinctive metallic green sheen (due to the Metachromatic properties of the dyes, *E. coli* movement using flagella, and strong acid end-products of fermentation). {Take 1 ml of diluent (1:10) pour in the LST broth and incubate at 35 °C for 24-48 h. if acid gas produce that it LST positive take one loop of LST positive culture inoculate in EC brothwith MUG

  supplement and incubate at 45°C for 24 hour. If acid gas produce that means EC positive take one loop culture of EC positive tube and streak on EMB agar and incubate at 37 °C for 24 h}.
- If green metallic sheen appear on EMB agar that means *E.coli* positive}.
- On the targtol 7 agar yellowish colonish are present so E.coli is positive.
- On the macconky agar E.coli give pink colour colonies present so E.coli is positive.

The test is positive on selective agar media proceed for the conformation of E.coli strike on nutrient agar plates minimum 5 Suspactive Colonies from the selective medium .

10.0 CONFERMATION OF E.coli:-

- **Test for Indole:-**Inoculate the loofull culture in a paptone water tube form the nutrient agar plate and incubate at 37°C for overnight**.** After incubation

add the kovac reagent in a peptone water tube shake the tube gently, the .appearance of a red colour indicates the presence of indole.

- **Test for H2S Production –** Prepare the TSI agar slant and inoculate the culture of E.coli from nutrient agar plate and incubate at37 °and observe daily for up to 7 days. The presence or absence of blackening in the butt of the medium shall be recorded.
- **Oxidase Test:-** Take the oxidase disk in a sterile place(LAF) and take the culture of E.coli from the nutrient agar plate. And rab on oxidase disk with the help so sterile wooden stick after 10 second colure is not change so E.coli is positive.
- **Motility Test: -** for the motility test prepare the motility agar tube and pierce/ insert the culture with the help of needle or loop from the nutrient agar plate. And incubate at 37°C for 18 hour. Motile strains shall be found to show growth on the surface of the medium outside the 'inner glass tube' having travelled through the entire medium inside this inner tube. If negative on the first day, keep the inoculated tube at room temperature for a further 4 to 6 days to see if evidence of motility is present.

11.0 Determination of the Most Probable Number of E.coli :-

- Obtain serial, with a fresh sterile pipette, a measured volume of 1 ml of the homogenized mixture and of the five following serial-dilutions of both dilution series in triplicate to the tubes of 10 ml of single strength MacConkey broth medium containing Durham's tube for collection of gas.
- Start with highest dilution and proceed to the lowest, filling and emptying the pipette three times before transferring the 1 ml portions to the tubes of medium.
- When the number of E.coli is assumed to be very small, start by transferring 10 ml of the homogenized mixture in triplicate to 10 'ml of double strength MacConkey broth medium containing Durham's tube for collection of gas, using a sterile 10 ml pipette.
- Incubate in a water-bath at 44oC for 48 hours.
- Examine the tubes showing production of acid and gas, and using Table 1, obtain the most probable number (MPN) of E.coli per gram of the sample.

- Use for the calculation the results from three dilutions, selecting the highest' dilution showing three positive tubes below which no sets with a smaller number of positive tubes occur, and the two following higher dilutions. .
- The number obtained from Table 1 has to be multiplied by the lowest dilution factor, namely that of the first set of tubes, to obtain the most probable number of E. coli per gram of the sample.
- Write the result from the MPN table(on the basis of positive or negative tube).

Annex A
(normative)

Determination of most probable number

See table A.1 and table A.2.

Table A.1 — MPN indexes and confidence limits

Number of positive results			MPN[1] index	Category[2] when number of tests is					Confidence limits[1][3]			
				1	2	3	5	10	⩾ 95 %		⩾ 99 %	
0	0	0	< 0,30						0,00	0,94	0,00	1,40
0	0	1	0,30	3	2	2	2	1	0,01	0,95	0,00	1,40
0	1	0	0,30	2	1	1	1	1	0,01	1,00	0,00	1,60
0	1	1	0,61	0	3	3	3	3	0,12	1,70	0,05	2,50
0	2	0	0,62	3	2	2	2	1	0,12	1,70	0,05	2,50
0	3	0	0,94	0	0	0	0	3	0,35	3,50	0,18	4,60
1	0	0	0,36	1	1	1	1	1	0,02	1,70	0,01	2,50
1	0	1	0,72	2	2	1	1	1	0,12	1,70	0,05	2,50
1	0	2	1,1	0	0	0	3	3	0,4	3,5	0,2	4,6
1	1	0	0,74	1	1	1	1	1	0,13	2,00	0,06	2,70
1	1	1	1,1	3	3	2	2	2	0,4	3,5	0,2	4,6
1	2	0	1,1	2	2	1	1	1	0,4	3,5	0,2	4,6
1	2	1	1,5	3	3	3	3	2	0,5	3,8	0,2	5,2
1	3	0	1,6	3	3	3	3	2	0,5	3,8	0,2	5,2
2	0	0	0,92	1	1	1	1	1	0,15	3,50	0,07	4,60
2	0	1	1,4	2	1	1	1	1	0,4	3,5	0,2	4,6
2	0	2	2,0	0	3	3	3	3	0,5	3,8	0,2	5,2
2	1	0	1,5	1	1	1	1	1	0,4	3,8	0,2	5,2
2	1	1	2,0	2	2	1	1	1	0,5	3,8	0,2	5,2
2	1	2	2,7	0	3	3	3	3	0,9	9,4	0,5	14,2
2	2	0	2,1	1	1	1	1	1	0,5	4,0	0,2	5,6
2	2	1	2,8	3	2	2	2	1	0,9	9,4	0,5	14,2
2	2	2	3,5	0	0	0	0	3	0,9	9,4	0,5	14,2
2	3	0	2,9	3	2	2	2	1	0,9	9,4	0,5	14,2
2	3	1	3,6	0	3	3	3	3	0,9	9,4	0,5	14,2
3	0	0	2,3	1	1	1	1	1	0,5	9,4	0,3	14,2
3	0	1	3,8	1	1	1	1	1	0,9	10,4	0,5	15,7
3	0	2	6,4	3	3	2	2	2	1,6	18,1	1,0	25,0
3	1	0	4,3	1	1	1	1	1	0,9	18,1	0,5	25,0
3	1	1	7,5	1	1	1	1	1	1,7	19,9	1,1	27,0
3	1	2	12	3	2	2	2	1	3	36	2	44
3	1	3	16	0	0	0	3	3	3	38	2	52
3	2	0	9,3	1	1	1	1	1	1,8	36,0	1,2	43,0
3	2	1	15	1	1	1	1	1	3	38	2	52
3	2	2	21	2	1	1	1	1	3	40	2	56
3	2	3	29	3	3	3	2	2	9	99	5	152
3	3	0	24	1	1	1	1	1	4	99	3	152
3	3	1	46	1	1	1	1	1	9	198	5	283
3	3	2	110	1	1	1	1	1	20	400	10	570
3	3	3	> 110									

1) From De Man, J. C., *Eur. J. Appl. Microbiol. Biotechnol.*, **17**, 1983, pp. 301-305.

2) See table A.2.

3) The confidence limits given in this table are meant only to provide some idea of the influence of statistical variations on results. There will always also be other sources of variation, which may sometimes be even more important.

12.0 TESTING FREQUENCY:-

12.1 For raw material per batch.

12.2 For online material; every lot (In process).

12.3 For finished material every batch.

13.0 ATTACHMENT -Nil

1.0 PURPOSE:-Detection and Enumeration of COLIFORM in food product.

2.0 SCOPE:-Detection and Enumeration of COLIFORM in food product.

3.0 RESPONSIBILITY:-Microbiologist.

4.0 REFERENCES:-IS 5401 (part 2):2002 /ISO 4831:1991

5.0 DEFINITIONS:-Coli form bacteria which at specified temperature (i.e. 30°C 35°C or 37°C) as agreed from characteristic colonies (purplish red) on violet red bile agar under the test condition.

6.0 AAPRATUS AND GLASSWARE:-Petri plate, Incubator, Autoclave, Test-tube, Water Bath, pH Meter, Colony Counting, Equipment, Laminar Air Flow Chamber etc.

7.0 CULTURE MEDIA:-Violet red bile agar (M049) final pH at 25 °C 7.4± 0.2 buffer peptone water (M 614) final pH at 25°C 7.2± 0.2, Brilliant Green Bile Broth 2% (M121) final pH at 25 °C 7.2± 0.2 sterilization is carried out by autoclave at 121°C for 15 minutes at 15 lbs.

8.0 PROCEDURE:-Test portion initial suspension and dilution –

Take 10 gm of sample and 90 ml of diluents buffer peptone water.

9.0 Detection (Absent/present) of Coliform:-

- Firstly make the suspension (10gm sample+90mlBPW) for detection of coliform
- Then prepare the LST tubes with durum's tube inoculate the 1 ml sample in a LST tube and incubate at 37°C for 24 hour.
- The gas formation indicate the presumptive test is positive because the fermentation of lactose.

- Than make the EC broth with durum's tube and inoculate the loofull culture in an EC broth tube and incubate at 37°C for 18 to20 hour.
- If the gas production is not present again incubate 24 hour.
- If the gas production is positive proceed for confirmation of coliform.

- Make the BGLB broth tube with durum's tube and inoculate the loop full culture from EC broth tube and incubate at 37°C for 18 to20 hour.
- If the gas production in tube and change the colure (found turbidity) of BGLB is called coliform is present.

10.0 Enumeration of coliform by MPN method:-

- For the Enumeration of coliform make the multiple tube of LST broth with durams tube (15 tubes).
- Each tube has 10ml LST broth.
- Then make the suspension of sample for MPN (10gm sample +90ml BPW).
- Then in the (1st) 3 tube (LST broth) 10ml sample are inoculated from the suspension of sample.
- Then in the (2ed) 3 tube (LST broth) 1ml sample are inoculated from the suspension of sample.
- Then in the (3ed) 3 tube (LST broth) 0.1ml sample are inoculated from the suspension of sample. And all tube are incubated at37°C for 24 hour.
- After incubation in tube gas production and turbidity and found so proceed for confirmation of coliform. (But if the gas production and turbidity not found in tube incubate again 24 hours).

11.0 Conformation stage:-Then make the 15 BGLB tube with durams tube (10ml) and inoculated the each dilution tube (positive /negative) in the BGLB broth tubes and

incubate all the tubes at 37°C for 24 hour. After incubation gas formation and turbidity found in a dilution tube (10ml, 1ml, 0.1ml) of BGLB.

12.0 Computing and recording of MPN:-The calculated density of coliform bacteria in a sample can be obtained from the MPN table, based on the number of positive tubes in each dilution of the confirmed test.

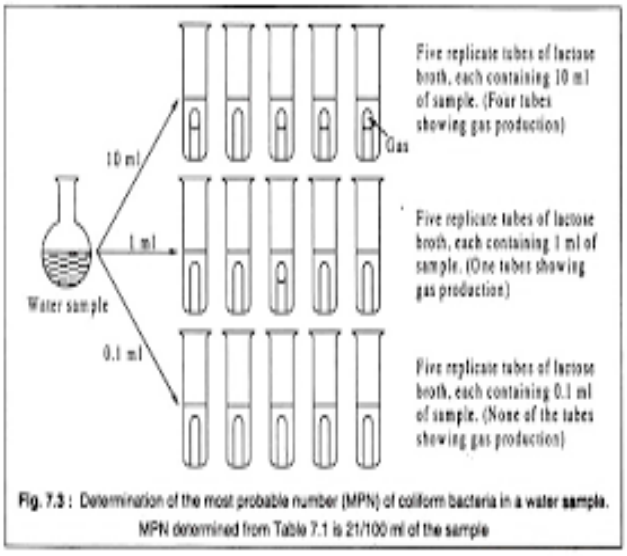


Fig. 7.3 : Determination of the most probable number (MPN) of coliform bacteria in a water sample. MPN determined from Table 7.1 is 21/100 ml of the sample

MPN DETERMINATION FROM MULTIPLE TUBE TEST

NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			MPN INDEX	95 PERCENT CONFIDENCE LIMITS	
3 of 10 ml each	3 of 1 ml each	3 of 0.1 ml each	per 100 ml	LOWER	UPPER
0	0	1	3	<0.5	9
0	1	0	3	<0.5	13
1	0	0	4	<0.5	20
1	0	1	7	1	21
1	1	0	7	1	23
1	1	1	11	3	36
1	2	0	11	3	36
2	0	0	9	1	36
2	0	1	14	3	37
2	1	0	15	3	44
2	1	1	20	7	89
2	2	0	21	4	47
2	2	1	28	10	150
3	0	0	23	4	120
3	0	1	39	7	130
3	0	2	64	15	380
3	1	0	43	7	210
3	1	1	75	14	230
3	1	2	120	30	380
3	2	0	93	15	380
3	2	1	150	30	440
3	2	2	210	35	470
3	3	0	240	36	1300
3	3	1	460	71	2400
3	3	2	1100	150	4800

FROM: STANDARD METHODS FOR THE EXAMINATION OF WATER AND WASTEWATER, TWELFTH EDITION. (NEW YORK: THE AMERICAN PUBLIC HEALTH ASSOCIATION, INC., p. 608)

13.0 Enumeration of coliform by plate count method:-

- Take two sterile Petri dishes using a sterile micropipette tips transfer to each dish 1 ml of the test sample if the product is liquid or 1ml of the initial suspension in the case of other products.
- Take two other sterile Petri dishes using a fresh sterile micro tips transfer to each dish 1 ml of the first decimal dilution (10^{-1}) of the test sample if the product is liquid or 1 ml of the first decimal dilution (10^{-2}) of the initial suspension in the case of other products repeat the procedure with the further dilutions using a fresh sterile micro tips for each decimal dilution.

- Pour about 15 ml of the VRBL medium at 45 °C ±0.5 °C in to each Petri dish the time eclipsing between the ends the preparation of the initial suspension (or of the 10^{-1} dilution if the product is liquid).
- Invert the prepared dishes and invert them in the incubator set 30°C, 35°C, and 37°C as agreed for 25°C for 2 hrs.

14.0 COUNTING THE COLONIES:-After the specified period of inoculation count using the colony counting unit equipments the colonies in each dish containing not more than 300 colonies.

15.0 EXPRESSION OF RESULT:-

- General case dishes containing between 15 and 300 colonies.
- Retained dishes containing not more than 300 colonies at two consecutive dilutions it is necessary that one of these dishes contain at least 15 colonies.
- Calculate the number N of microorganism per milliliter or per gram of the product depending on the case using the following equation.

$$N = \frac{\Sigma C}{(n_1 + 0.1 n_2)\, d}$$

Where

ΣC is the sum of colonies contends on all the dishes retained.

n_1 is the number of dishes retained in the first dilution.

n_2 is the number of dishes retained in the second dilution.

d is the dilution factor corresponding to the first dilution.

Round the result calculated to two significance figures

Take as the number of microorganisms per milliliter or per gram of product expressed as a number between 1.0 and 9.9 multiplied by 10^X is the appropriate power of 10

16.0 CONFIRMATION OF COLIFORM:-Take suspected a lapful colony in BGLB tube and incubate at 30°C or 37±1 for 25 h gas formation that is colifrom positive.

17.0 TESTING FREQUENCY:-

- For raw material per batch.
- For online material; every lot (Inprocess).
- For finished material every batch.

18.0 ATTACHMENT -Nil

1.0 PURPOSE:-Method for Isolation and Detection and Identification of STAYPHYLOCOCCUS AUREUS (responsible for food poisoning).

2.0 SCOPE:-Isolation and Detection and Identification of STAYPHYLOCOCCUS AUREUS (responsible for food poisoning).

3.0 RESPONSIBILITY:-Microbiologist.

4.0 REFERENCES:-IS 5887/ISO 6888-3:2003

5.0 DEFINITIONS:-Staphylococcus aurous are aerobes or facultative gram positive no motile cocci growth of s. aureus ranges from 7 to 47.8°C with an optimum temperature of 35°C the pH ranges for growth is between 4.5 and 9.3 with the optimum between pH 7.0 and 7.5 the bacterium is also highly salt tolerant resistant to nitrites and capable of growth at aw values as low as 0.83 under ideal conditions.

6.0 AAPRATUS AND GLASSWARE:-Petri Plate, Incubator, Autoclave, Test-tube, Water bath pH meter, Colony Counting Equipment, Vortex mixture, Laminar Air Flow Chamber, Inoculating loop etc.

7.0 CULTURE MEDIA:- Giolitti and cantoni broth (M5841),Pttasium Tallurite (FD052), Paraffin wax pellets(GRM10702), Brain Heart Infusion Broth(LQ077),Baired parker agar (M043) final pH at 25 °C 7.0± 0.2 sterilization is carried out by autoclave at 121°C for 15 minutes at 15 lbs after sterilization aseptically add Egg yolk telluride emulsion (FD046), Buffer peptone water (M614) final pH 7.2±0.2 sterilization is carried out by autoclave at 121°C for 15 minutes at 15 lbs.

8.0 PROCEDURE-

- **Detection of S.aureus:-**

8.1 Presumptive Test:-

- Firstly make the suspension (10gm sample +90ml BPW).
- Then take the giolitti and cantoni broth with potassium tallurite supplement. And inoculate the 1ml of the sample in a Giolitti Cantoni broth tube and seal the tube with paraffin.Then incubate the tube at 37°C for 24 hour. (After incubation the test is negative reincubate negative tubes up to 48 hour.).
- After incubation change the colure of gioltti cantoni broth show black in colour.
- For isolation of S.aureus streaked on Baird Parker agar from the giolitti cantoni broth tube and incubate at 37°C for 24 hour.
- After incubation shows black colonies surrounded by clear zones.
- For the conformation test subculture the colonies on Nutrient agar media from the Baird Parker agar media and incubate 37°C for overnight.

9.0 CONFIRMATION OF S. AUREUS:-

9.1 On Blood Agar:-

- After incubation separated number of colonies strikes on Blood agar media and incubates 37°C for overnight.
- After incubation beta Hemolysis is present with zone oh hemolysis by beta-toxin on blood agar is called S. aureus is present.

9.2 By Brain Heart Infusion Test:-

- A Coagulase test Most of the S.aureus strains are coagulate positive although in very few cases it could be coagulate negative.
- Test tube containing 5 ml of Brain heart infusion broth is inoculated with the material being tested Incubate for 24 h at 37°C.
- Add 2-3 drops of the culture to a hemolytic tube containing 0.5 ml rabbit plasma.
- Incubate at 37 °C periodically observing coagulation usually take place before 4 hour of inoculation.
- If formation of clump it means S. aureus positive.

9.3 Catalase Test –

- Most of the s. aureus strain are catalase positive although in very few cases it could be catalase negative.
- Catalases emulsify a colony in a drop of hydrogen peroxide on a glass slide immediate bubbling indicates a positive catalase test.

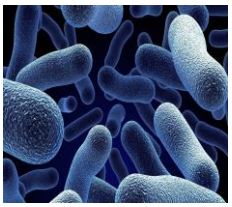
10.0 TESTING FREQUENCY:-

10.1 For raw material per batch.

10.2 For online material; every lot (In process).

10.3 For finished material every batch.

11.0 ATTACHMENT –Nil



Microbiology Method On The basis of IS and ISO Standard

TITLE: SOP FOR MICROBIOLOGY LAB.			
Document Name:	Standard Operating Procedure	Effective Date	09/11/2017
Document Number		Issue Date	
Department:	Quality control- Microbiology		

1.0 PURPOSE- Method for Isolation and Detection and Identification of SALMONELLA (responsible for food poisoning).

2.0 SCOPE:-Isolation and Detection and Identification of SALMONELA (responsible for food poisoning).

3.0 RESPONSIBILITY: Microbiologist.

4.0 REFERENCES: IS 5887 (part 3):1999/ISO6579:1993

5.0 DEFINITIONS:-The typical salmonella is aerobic, gram negative rod motile, salmonella microorganism which form typical colonies on solid selective media and which display the biochemical and serological characteristics describe when test carried out in according to that test method.

6.0 AAPRATUS AND GLASSWARE:-Petri plate, Incubator, Autoclave, Test-tube, Water bath, pH Meter, laminar Air Flow Chamber, Vortex mixture etc.

7.0 CULTURE MEDIA:-XLD medium (Xylose Lysine decarboxilase agar) (M031) final pH at 25°C 7.0±0.2 do not autoclave overheat , Buffer peptone water(M614), Urea Broth(M111)Triple Sugar-Iron Agar Medium (MM021), Rappaport Vassiliadis Medium (M880) Selenite cystine medium fluid(M025), final pH 7.2±0.2 do not autoclave.

8.0 PROCEDURE:-

8.1 Pre enrichment in non selective liquid medium –

8.1.1 Take 25 gm sample and 225 ml buffer peptone water incubation of buffer peptone water (also use as diluents) with test portion and incubation at 35 °C or 37 °C (as agreed) for 16-20 hours.

8.2 Enrichment in selective liquid medium –

8.2.1 Incubation on are Rappaport –Vassiliadis medium with the culture obtain from 8.1.1

8.2.2 Incubation of Rappaport –Vassiliadis at 35 °C or 37 °C (as agreed FOR 24 Hours).

(Enrichment in selective liquid medium Inoculation of a fluid Selenite Cystin medium with the culture obtained in 8.1.1 inoculation of the fluid Selenite Cystin medium at 35 °C or 37 °C (as agree for 24 Hours).

Prepared By:- Mr. Vinay Katare	**Checked By: Mr. Abhishek Garhewal**	**Approved By:-**

8.3 Sticking on Solid Selective Medium –

8.3.1 Take 1 loop culture streak on XLD agar (From Selenite Cystin medium), incubate at 37 C for 24 hours.

8.3.2 If aware on the XLD Agar - Pink colonies with or without black centers. Many Salmonella colonies may have large, glossy black centers or may appear as almost completely black colonies. Atypically, a few Salmonella cultures produce yellow colonies with or without black centers.

8.4 Selection of colonies for confirmation:-

8.4.1 For confirmation, take from each dish of each selective medium five colonies considered to be typical or suspect.

8.4.2 If on one dish there are fewer than five typical or suspect colonies, take for confirmation all the typical or suspect colonies.

8.4.3 Streak the selected colonies onto the surface of predried nutrient agar plates in a manner which will allow well-isolated colonies to develop.

8.4.4 Incubate the inoculated plates at 35°C or 37°C (as agreed) for 18 h to 24 h.

8.4.5 Use pure cultures for biochemical and serological confirmation.

9.0 CONFERMATION OF SALMONELLA:-

9.1 Biochemical Test:- Striking on TSI agar slant…

9.1.1 Streak the agar slope surface and stab the butt.

9.1.2 Incubate at 35°C or 37°C (as agreed) for 24 h.

Interpret the changes in the medium as follows:-

- Butt Yellow: - Glucose positive (Fermentation of Glucose).
- Red or Unchanged: - Glucose Negative (no Fermentation of Glucose).
- Black: - Formation of Hydrogen sulfide.
- Bubbles or Cracks: - Gas formation from Glucose.
- Slant Surface:-
- Yellow: - Lactose / Sucrose Positive (Lactose/ Sucrose used).

- Red or Unchanged: Lactose and Sucrose negative (neither Lactose nor Sucrose used).

Typical Salmonella cultures show alkaline (red) slants with gas formation and acid (yellow) butts, with (in about 90 % of the cases) formation of hydrogen sulfide (blackening of the agar).

When lactose-positive Salmonella is isolated the TSI slant is yellow. Thus, preliminary confirmation of Salmonella cultures shall not be based.

9.2 Urease Test:

9.2.1 Transfer all presumed-positive cultures grown on TSI slants to tubes of urea broth and incubate at 35-37 °C for 22-26 hrs.

9.2.2 Cultures that are urease positive (purple-red), regardless of other reactions, are not Salmonella suspects. Retain all urease negative cultures for further study.

9.3 L-Lysine Decarboxylation Medium: -

9.3.1 Inoculate just below the surface of the liquid medium.

9.3.2 Incubate at 35°C or 37°C (as agreed) for 24 h.

9.3.3 A purple colour after incubation indicates a positive reaction.

9.3.4 A yellow colour indicates a negative reaction.

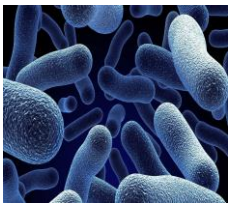
9.4 IMViC Test:-

- Indole:-Positive
- Methyle Red:-Positive
- Voges–Prosker Test:-Negative
- Simmons Citrate:-Positive

10.0 TESTING FREQUENCY:-

- For raw material per batch.
- For online material; every lot (In process).
- For finished material every batch.

11.0 ATTACHMENT –Nil



Microbiology Method On The basis of IS and ISO Standard

TITLE: SOP FOR MICROBIOLOGY LAB.			
Document Name:	Standard Operating Procedure	Effective Date	09/11/2017
Document Number		Issue Date	
Department:	Quality control- Microbiology		

1.0 Purpose: - Method for the Enumeration of viable Bacillus Cereus by Colony counts Technique.

2.0 Scope: - Method for the Enumeration of viable Bacillus Cereus by colony Count Technique.

3.0 Responsibility: - Microbiologist.

4.0 Reference: - IS 5887(Part 6):2012/ISO7932:2004

5.0 Definition:-Bacillus cereus is a Gram-positive, rod shaped, aerobic, motile, beta hemolytic bacterium commonly found in soil and food. Some strains are harmful to humans and cause food borne illness, while other strains can be beneficial as probiotics for animals. It is the cause of "fried rice syndrome", as the bacteria are classically contracted from fried rice dishes that have been sitting at room temperature for hours. B. cereus bacteria are facultative anaerobes, and like other members of the genus Bacillus, can produce protective endospores. Its virulence factors include cereolysin and Phospholipase C.

6.0 APPARATUS AND GLASSWARE:- Petri plate, Incubator, Autoclave, Test-tube, Water Bath pH Meter, Colony Counting Equipment, Laminar Air Flow Chamber, L-Shaped Spreader etc.

7.0 CULTURE MEDIA:- Bacillus Cereus Agar Base (M833) Final pH (at 25°C) 7.2±0.2,Egg yolk emulation (FD045)**,** Polymyxin B solution(FD003)**,**Nutrient agar(M001),MYP AGAR BASE(M636F) Final pH (at 25°C) 7.2±0.2. Sterilization is carried out by autoclave at 121 °C for 15 minutes at 15 lbs.

8.0 PROCEDURE:-

8.1 Sample preparation:-

Test portion initial suspension and dilution:-

- Take 10 gm of sample and 90 ml of diluents buffer peptone water.

8.2 Plate count of B. cereus:-

8.2.1 Inoculate duplicate Bacara or MYP agar plates with each dilution of sample (including 1:10) by spreading 0.1 mL evenly onto surface of each plate with sterile glass spreading rod.

8.2.2 Incubate plates 18-24 h at 30°C and observe for colonies surrounded by precipitate zone, which indicates that lecithinase is produced. *B. cereus*colonies are usually a pink-orange color on Bacara or pink on MYP and may become more intense after additional incubation.

8.2.3 If reactions are not clear, incubate plates for additional 24 h before counting colonies. Select plates that contain an estimated 15-150 pink-orange (Bacara) or pink (MYP), lecithinase-producing colonies.

8.2.4 Mark bottom of plates into zones with black felt pen to facilitate counting and count colonies that are typical of *B*. *cereus*.

8.2.5 This is the presumptive plate count of *B*. *cereus*.

8.2.6 Pick at least 5 presumptive positive colonies from the Bacara or MYP plates and transfer one colony to BHI with 0.1% glucose for enterotoxin studies and a nutrient agar slant for storage.

8.2.7 Typical colonies grown on Bacara or MYP must be confirmed with biochemical testing.

8.2.8 Calculate number of *B*. *cereus* cells/g of sample, based on percentage of colonies that are morphologically consistent with *B*. *cereus*.

9.0 Confirmation of B. cereus:-

9.1 Pick 5 or more eosin pink, lecithinase-positive colonies from MYP agar plates and transfer to nutrient agar slants.

9.2 Incubate slants 24 h at 30°C.

9.3 Prepare Gram-stained smears from slants and examine microscopically.

9.4 B. cereus will appear as large Gram-positive bacilli in short-to-long chains; spores are ellipsoidal, central to subterminal, and do not swell the sporangium.

9.5 Transfer 3 mm loopful of culture from each slant to 13 × 100 mm tube containing 0.5 ml of sterile phosphate-buffered dilution water and suspend culture in diluent with Vortex mixer.

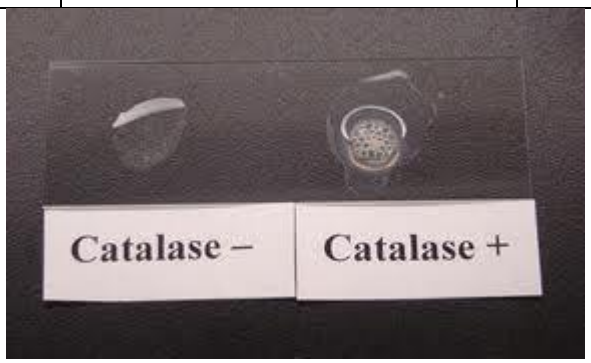
Use suspended cultures including ATCC 14579 B. cereus and ATCC 64 Brevibacillus laterosporus as positive and negative controls respectively to inoculate the following confirmatory media:-

9.1.1 Phenol red Glucose Broth:-

- Inoculate 3 ml broth with 2 mm loop full of culture.
- Incubate tubes anaerobically 24 h at 35°C in GasPak anaerobic jar.
- Shake tubes vigorously and observe for growth as indicated by increased turbidity and color change from red to yellow, which indicates that acid has been produced anaerobically from glucose.
- A partial color change from red to orange/yellow may occur, even in uninoculated control tubes, due to a pH reduction upon exposure of media to CO_2 formed in Gas Pak anaerobic jars.

9.1.2 Catalase Test:-

- Transfer a small amount of bacterial colony to a surface of clean, dry glass slide using a loop or sterile wooden stick.
- Place a drop of 3% H_2O_2 on to the slide and mix.
- A positive result is the rapid evolution of oxygen (within 5-10 sec.) as evidenced by bubbling.
- A negative result is no bubbles or only a few scattered bubbles.
- Dispose of your slide in the biohazard glass disposal container.



9.1.3 Haemolysis Test on Sheep Blood Agar:-

- Streak the selected colonies on nutrient agar plate on to the surface of sheep blood agar in a manner which allows good interpretation of the haemolysis reaction.
- Incubate at 30 °C for 24 h ± 2 h and interpret the haemolysis reaction.

10.0 EXPRESSION OF RESULT:-

General case dishes containing between 15 and 300 colonies.

Retained dishes containing not more than 300 colonies at two consecutive dilutions it is necessary that one of these dishes contain at least 15 colonies. Calculate the number N of microorganism per milliliter or per gram of the product depending on the case using the following equation.

$$N = \frac{\Sigma C}{(n_1 + 0.1 n_2).d}$$

Where

- Σc is the sum of colonies contends on all the dishes retained.
- n_1 is the number of dishes retained in the first dilution.
- n_2 is the number of dishes retained in the second dilution.
- d is the dilution factor corresponding to the first dilution.

- Round the result calculated to two significance figures.
- Take as the number of microorganisms per milliliter or per gram of product expressed as a number between 1.0 and 9.9 multiplied by 10X is the appropriate power of 10gm.

11.0 TESTING FREQUENCY:-

- For raw material per batch
- For online material; every lot (In process)
- For finished material every batch.

12.0 ATTACHMENT:–Nil

1.0 Purpose: - Method for Detection and Enumeration of Listeria Monocytogenes.

2.0 Scope: - Method for Detection and Enumeration of Listeria Monocytogenes.

3.0 Responsibility: - Microbiologist.

4.0 References: - IS 14966 **(part 1): 2001**

5.0 Definitions:-Listeria Monocytogenes is a Gram-positive, non spore-forming, motile, facultatively anaerobic, rod-shaped bacterium. It is catalase-positive and oxidase-negative, and expresses a beta Hemolysin, which causes destruction of red blood cells. This bacterium exhibits characteristic tumbling motility when viewed with light microscopy. Although L. Monocytogenes is actively motile by means of peritrichous flagella at room temperature (20–25 °C), the organism does not synthesize flagella at body temperatures (37 °C) Its ability to grow at temperatures as low as 0 °C permits multiplication at typical refrigeration temperatures, greatly increasing its ability to evade control in human foodstuffs.

6.0 Apparatus and Glassware:-Petri plate, Incubator, Autoclave, Test-tube, Water Bath, pH Meter, Colony Counting Equipment, Laminar Air Flow Chamber, Inoculating Loop, Micro Pipette etc.

7.0 Culture Media: - Half Fraser Broth, Fresher Broth (M1327) Final pH (at 25°C) 7.2±0.2, Oxford Agar (M1145) Final pH (at 25°C) 7.0±0.2, Palcam Agar (Final pH (at 25°C) 7.0±0.2. Tryptone Soya Yeast Extract Agar (TSYEA), Sheep Blood Agar.

8.0 Procedure:-

8.1 Test portion, Initial Suspension:-

Take the 25gm sample and 225ml Half Fresher Broth.

8.2 Pre-Enrichment in non Selective Liquid Medium:-

Primary enrichment in a Selective Liquid Enrichment Medium with reduced concentration of selective agents (Half Fraser Broth 225ml) and make the suspension of (25×225) sample and incubate at 37°C for 24 hours.

8.3 Enrichment in Selective Liquid Medium:-

Secondary enrichment with a Selective Liquid Enrichment Medium with full concentration of selective agents (Fraser broth then take the fresher broth 10ml tubes, take the loop full sample from half fresher broth in a secondary medium(Fresher Broth) and incubate at 37°C for 24 hours.

8.4 Sticking on solid selective medium:-

After incubation of Fresher Broth tube take the oxford and Palcam Agar Media. From the cultures obtained in plating out on the two selective solid media:

- **Oxford Agar**:-Take the lapful culture of Fresher Broth tube and strike on the Oxford Media and incubate the plats invertly at 37°C.

- **Palcam Agar**:-Take the loopful culture of Fresher Broth tube and strike on the Palcam Media and incubate the plats invertly at 37°C. After incubation for 24 h and for an additional 18 h to 24 h (if growth is slight or if no colonies are observed after 24 h of incubation), examine the dishes for the presence of colonies presumed to be Listeria spp.

9.0 Expression of Result:-

- **Oxford Agar:** Typical colonies of Listeria spp. grown on Oxford Agar for 24 hours are small (1 mm) Grayish Colonies surrounded by black halos. After 48 hours colonies become darker, with a possible greenish sheen, and are about 2 mm in diameter, with black halos and sunken centers.
- **PALCAM Agar**: For plates incubated micro aerobically, after incubation expose the PALCAM Agar plates to air for 1 hour to allow the medium to regain its pink to purple colour. After 24 hours Listeria spp. grow as small or very small grayish green or olive green colonies, 1.5 mm to 2 mm in diameter, sometimes with black centers, but always with black halos. After 48 hours. Listeria spp. appear in the form of green

colonies about 1.5 mm to 2 mm in diameter, with a central depression and surrounded by a black halo.

10.0 Confirmation of Listeria spp.:-

10.1 Selection of colonies for Confirmation:-

For confirmation, take from each plate of each selective medium five colonies presumed to be Listeria spp. If on one plate there are fewer than five presumed colonies, take for confirmation all of them. Streak the selected colonies onto the surface of pre-dried plates of Tryptone Soya Yeast Extract Agar (TSYEA) in a manner which will allow well- separated colonies to

develop. Place the plates in the incubator set at 35 ‘C or 37 ‘C for 18 hours to 24 hours or until growth is satisfactory.

The temperature of the inoculated medium should be agreed upon between the parties concerned and recorded in the test report. Typical colonies are 1 mm to 2 mm in diameter, convex, colorless and opaque with an entire edge. If the colonies are not well separated, pick a typical Listeria spp. colony onto another TSYEA plate. Carry out the following tests from colonies of a pure culture on the TSYEA.

10.2 Catalase Reaction:-Take an isolated colony obtained in and suspend it in a drop of hydrogen peroxide solution on a slide. The immediate formation of gas bubbles indicates a positive reaction.

10.3 Gram Staining:-

Perform the Gram Stain on a colony separated in Listeria spp. is revealed as Gram-positive slim, short rods.

10.4 Motility Test:-

Take an isolated. Colony obtained in and suspends it in a tube containing TSYEB. Incubate in the incubator set at 25 “C for 8 hours 24 hours until a cloudy medium is observed. Deposit a drop of the above culture using a loop

onto a clean glass microscope slide. Place a cover slip on top and examine it with the microscope Listeria spp. appears as slim, short rods with tumbling motility. Cultures grown above 25°C may fail to exhibit this motion. Always compare them to a known culture. Cocci, large rods, or rods with rapid swimming motility are not Listeria spp. As an alternative test for motility, using an inoculating Needle stab the motility agar with a culture taken from a typical! Colony on TSYEA incubates it for 48 h in the incubator set 25 “C.

Examine for growth around the stab. Listeria spp. Are motile, giving a typical umbrella-like growth pattern. If growth is not sufficient, incubate for up to an additional 5 days andobsewe the stab again.

10.5 Haemolysis Test:-

If the morphological and physiological characteristics and catalase reaction are indicative of Listeria SPP inoculate the Sheep Blood Agar plates to determine the haemolytic reaction.

11.0 TESTING FREQUENCY:-

- For raw material per batch.
- For online material; every lot (In process).
- For finished material every batch.

12.0 ATTACHMENT:-Nil

1.0 Purpose:- Method for Enumeration(MPN) and Colony Count Technique of Enterobacteriaceae.

2.0 Scope:- Method for Enumeration(MPN) and Colony Count Technique of Enterobacteriaceae.

3.0 Responsibility:- Microbiologist.

4.0 References: - IS/ISO 7402:1993.

5.0 Definitions: Microorganisms which Ferment Glucose and show a Negative Oxides Reaction when the test is carried out according to the method specified.

6.0 APPARATUS AND GLASSWARE:- Petri Plate, Incubator, Autoclave, Test tube, Water Bath, pH Meter, Colony Counting Equipment, Inoculating loop, Laminar Air Flow Chamber, Micro Pipette etc.

7.0 Culture Media: - Buffered Brilliant Green Bile Glucose Broth, EE Broth (M287), Violet Red Bile Glucose Agar (VRBGA) (M581), Glucose Agar (M1589), Nutrient Agar (M001), Oxides Reagent.

8.0 Procedure:-

- Prepare a single decimal dilution series from the test sample if the product is liquid, or from the initial suspension in the case of other products.
- Take two sterile Petri dishes. Using a sterile pipette, transfer to each dish 1 ml of the test sample.
- If the product is liquid, or 1 ml of the initial suspension in the case of other products.
- Take two other sterile Petri dishes.
- Using a fresh sterile pipette, transfer to each dish 1 ml of the first decimal dilution (10- 1, of the test sample if the product is liquid, or 1 ml of the first decimal dilution of the initial suspension (10-2) in the case of other products.
- Repeat the procedure described with the further dilutions, using a fresh sterile pipette for each decimal dilution.

- Pour into each Petri dish approximately 15 ml of the VRBGA medium which has been prepared then cooled to approximately 45°C in the water bath.

- The time elapsing between the end of the preparation of the initial suspension (or of the 10-1 dilution if the product is liquid) and the moment when the medium is poured into the dishes shall not exceed 15 min.
- Carefully mix the inoculums with the medium by horizontal movements and allow the mixture to solidify, with the Petri dishes standing on a cool horizontal surface.
- After complete solidification of the mixture, add a covering layer of 10 ml to 15 ml of the VRBG medium prepared then cooled as described in to prevent spreading growth and to obtain semi-anaerobic conditions.
- Alloy to solidify as described above. Invert the prepared dishes and incubate them in the incubator set at 35 “C or 37 °C (as agreed) for 24 h.

9.0 General:-

- If at least 80 **%.** Of the selected typical colonies are oxides-negative and glucose-positive and Thus confirmation as Enterobacteriaceae.
- The number of microorganisms present will be the same as that given by the count made in.
- In all other cases, the number shall be calculated from the percentage of oxides-negative and glucose positive colonies in relation to the total number of selected colonies.
- Calculate the number, N, of Enterobacteriaceae per milliliter or per gram of product, using the following equation:

ΣC

$$N = \frac{\Sigma c}{(n_1 + 0.1 n_2).d}$$

Where

- Σc is the sum of colonies contends on all the dishes retained.
- n_1 is the number of dishes retained in the first dilution.
- n_2 is the number of dishes retained in the second dilution.
- d is the dilution factor corresponding to the first dilution.

10.0 Counting and selection of colonies:-

Select the dishes containing less than 150 typical colonies of diameter 0,5 mm or more; count these suspect colonies. Select at random five such colonies from each dish for biochemical confirmation after sub culturing. Consider the determination to be void if half or more than half the surface area of a dish is overgrown. If less than half of the surface area of a dish is overgrown, count the colonies on the clear part and extrapolate so that the number corresponds to the total surface area of the dish.

11.0 Selection of colonies for confirmation:-

From each of the plates incubated on which typical pink to red colonies (with or without Precipitation haloes) or colorless, mucoid colonies have developed, select at random five such colonies for biochemical confirmation after sub culturing.

12.0 Confirmation Test:-

12.1 Sub Culturing:-

Streak on nutrient agar plates each of the colonies selected for confirmation. Incubate these plates at 35 “C or 37 “C (as agreed) for 24 h + 2 h. Select a well-isolated colony from each of the incubated plates for biochemical confirmation.

Biochemical confirmation:-

12.2 Oxides Reaction:-

Using the platinum/iridium loop or wire or glass rod take a portion of each well-isolated colony and streak on a filter paper moistened with the oxides

reagent or on a commercially available disc. A nickel/chromium loop or wire shall not be used.
Consider the test to be negative when the colour of the filter paper has not turned dark in 10 s.
Consult the manufacturer's instructions for ready-to use discs.

12.3 Fermentation Test:-

Stab, using a wire the same colonies selected into tubes containing glucose agar. Incubate at 35 °C or 37°C (as agreed) for 24 h + 2 h. If a yellow colour develops throughout the contents of the tube, the reaction is regarded as positive. Strains produce gas.

13.0 MPN Technique:-

The Enumeration of Enterobacteriaceae by MPN Technique involves Six Stages:-

- Inoculation of three tube of bufferd peptone water per dilution of the test sample, using those dilution appropriate to obtanning the required detaction parameters for thet product.
- Incubation of those tube at 37°C for 24 hour.
- Subculture of Enterobacteriaceae Enrichment (EE) broth (do not Autoclave) with incubation at 37°C for 24 hours.
- Subculture (strike) of each tube to Violet Red Bile Glucose (VRBGA) and incubation at 37°C for 24 hours.
- Conforming of Enterobecteriaceae presence from tube producing red-purple colonies on VRBGA by glucose fermentation and Oxidase test.
- Determination of the MPN index from the number of positive tube of selected dilution using an MPN table and calculation of the Enterobecteriaceae count per gram or milliliter of sample.

Count the number of tubes giving a positive reaction for each Dilution:-

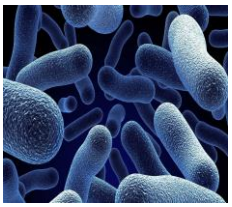
- If one of the selected typical colonies of a subculture is oxides-negative and glucose-positive, the tube from which the subculture is derived shall be regarded as being positive. Using the MPN table, determine from the number of positive tubes in the different dilutions, the most probable number (MPN) table.

- In the case of liquid products, the number of Enterobacteriaceae per milliliter is calculated by dividing the MPN table.
- In the case of other products for which initial suspensions are prepared, the number per gram is equal to the MPN index.

14.0 TESTING FREQUENCY:-

- For raw material per batch.
- For online material; every lot (In process).
- For finished material every batch.

15.0 ATTACHMENT: -Nil



Microbiology Method On The basis of IS and ISO Standard

TITLE: SOP FOR MICROBIOLOGY LAB.			
Document Name:	Standard Operating Procedure	Effective Date	09/11/2017
Document Number		Issue Date	
Department:	Quality control- Microbiology		

1.0 **Purpose:** - Method for Detection and Enumeration of Clostridium Perfringens.

2.0 **Scope**: - Method for Detection and Enumeration of Clostridium Perfringens.

3.0 **Responsibility: -** Microbiologist.

4.0 **References: -** IS 5887(part 4):1999.

5.0 **Definitions:** Clostridium Perfringens is the Gram positive,rod shaped, anaerobic, Typical food-poisoning strains of Clostridium perfringens are non-haemolytic on horse blood agar, and produce lecithinase as demonstrable on the egg-yolk plates in showing precipitates around the colonies The spores are heat resistant at 100°C for one hour however, some workers have reported reduced heat resistance of the spores when cultures are grown in Ellner's medium.

6.0 **APPARATUS AND GLASSWARE:-** Petri Plate, Incubator, Autoclave, Test tube, Water Bath, pH Meter, Colony Counting Equipment, Inoculating Loop, Anaerobic jar, Gas pack, Laminar Air Flow Chamber, CO_2 Indicator Tablet etc.

7.0 **Culture Media**:- Sheep Blood Agar (MP1301),Neomycin (FD174), Cooked Meat Medium(M149),Willis and Hobbs Medium (M1375), Nutrient Broth(MM244), Nutrient Agar (M001),

8.0 **Procedure for Absent\ Present:-**

8.1 The sample is blended in a sterile blender\jar for 2 minutes or macerated with sterile sand in a sterile mortar using approximately 225ml of diluting fluid per approximately 25g of the sample.

8.2 Should be 0.1 percent peptone in water sterilized at 120°C for 20 minute, final ph 6.8(-+) or 3.4 percent of potassium dihydrogen phosphate (KH_2PO_4) in water. PH adjusted to 7.2 and sterilized at 120°C. for 20ml.

8.3 An aliquot of the specimen is inoculated into cooked meat medium and the inoculated tube heated in a steamer at 100°C for one hour and incubated overnight at37°C.

8.4 An aliquot of the specimen is also inoculated directly on to blood agar medium and egg yolk medium and incubated in an anaerobic jar at 37°C overnight.

8.5 Subcultures are made from the growth in medium on to the two solid media and incubated in an anaerobic jar at 37°C overnight.

Prepared By:- Mr. Vinay Katare	**Checked By: Mr. Abhishek Garhewal**	**Approved By:-**

8.6 If the typical food –poisoning strains of clostridium perfringens are non haemolytic on horse blood agar and produse lecithinase as demonstrable on the egg yolk plates in showing precipitates around the colonies. So positive the clostridium perfringnens.

8.7 The spore are heat resistant at 100°C for one hour, however, some workers have reported reduced heat resistance of the spore when cultures are grown in ellner's medium.

9.0 ENUMERATION:-

9.1 The sample 25 to 50g of sample is taken in a sterile blender\jar and to this is added diluting fluid to have dilution of 10^{-1} .Blend at 8000 to 10000rev\min for 2 minute.

9.2 Alternatively macerate the sample with diluting fluid in a sterile mortar with sterile sand.

9.3 Make serial tenfold dilution with dilutions with the diluting fluid in duplicate series up to 10^{-7}.

9.4 Streak 0.1 ml from each tube evenly on to blood agar medium and also on egg yolk medium.

9.5 Incubate in an anaerobic jar at 37°C for 18 to 24 hours.

9.6 It is useful to incubate aerobically duplicate plates similarly inoculated for comparison. The suspect colonies are counted and the number of visible colonies per gram of sample determined by multiplying by the dilution factor and dividing by the mass of the sample.

10.0 Confirmation:-

After the incubation of cooked meat medium, the presumptive test is positive strike on the blood agar and willis and hobb's medium with neomycin and incubation anarobically the typical colonies on medium like non haemolytic on horse blood agar and produce lecithinase as demonstrable on the egg yolk plates in showing precipitates around the colonies. So positive colonies of clostridium perfringnens).

11.0 TESTING FREQUENCY:-

- For raw material per batch.
- For online material; every lot (In process).
- For finished material every batch.

12.0 ATTACHMENT: -Nil